Received: 1 September 2021 | Revised: 23 September 2021 | Accepted: 23 September 2021

DOI: 10.14814/phy2.15095

ORIGINAL ARTICLE

Physiological Reports

Blockade of the natriuretic peptide clearance receptor attenuates proteinuria in a mouse model of focal segmental glomerulosclerosis

Liming Wang[1] | **Yuping Tang**[1] | **Anne F. Buckley**[2] | **Robert F. Spurney**[1]

[1]Division of Nephrology, Department of Medicine, Duke University and Durham VA Medical Centers, Durham, North Carolina, USA

[2]Department of Pathology, Duke University Medical Center, Durham, North Carolina, USA

Correspondence
Robert F. Spurney, MSRB II, Room 2013, 106 Research Drive, Durham, NC, USA.
Email: robert.spurney@dm.duke.edu

Funding information
These studies were supported by the following grants: (1) W81XWH-19-1-0314 from the Department of Defense, and (2) BX002984 from the Veterans Administration Merit Review Program.

Abstract

Glomerular podocytes play a key role in proteinuric diseases. Accumulating evidence suggests that cGMP signaling has podocyte protective effects. The major source of cGMP generation in podocytes is natriuretic peptides. The natriuretic peptide clearance receptor (NPRC) binds and degrades natriuretic peptides. As a result, NPRC inhibits natriuretic peptide-induced cGMP generation. To enhance cGMP generation in podocytes, we blocked natriuretic peptide clearance using the specific NPRC ligand ANP(4-23). We then studied the effects of NPRC blockade in both cultured podocytes and in a mouse transgenic (TG) model of focal segmental glomerulosclerosis (FSGS) created in our laboratory. In this model, a single dose of the podocyte toxin puromycin aminonucleoside (PAN) causes robust albuminuria in TG mice, but only mild disease in non-TG animals. We found that natriuretic peptides protected cultured podocytes from PAN-induced apoptosis, and that ANP(4-23) enhanced natriuretic peptide-induced cGMP generation in vivo. PAN-induced heavy proteinuria in vehicle-treated TG mice, and this increase in albuminuria was reduced by treatment with ANP(4-23). Treatment with ANP(4-23) also reduced the number of mice with glomerular injury and enhanced urinary cGMP excretion, but these differences were not statistically significant. Systolic BP was similar in vehicle and ANP(4-23)-treated mice. These data suggest that: 1. Pharmacologic blockade of NPRC may be useful for treating glomerular diseases such as FSGS, and 2. Treatment outcomes might be improved by optimizing NPRC blockade to inhibit natriuretic peptide clearance more effectively.

KEYWORDS

cell signaling, focal segmental glomerulosclerosis, glomerular podocyte, natriuretic peptides

1 | INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is characterized by segmental scarring of the glomerulus, nephrotic syndrome, and often rapid progression to end stage kidney disease (ESKD; D'Agati et al., 2011). The incidence of FSGS has increased ~10-fold in the last 2–3 decades (Kitiyakara et al., 2004; Korbet, 2012), and the disease is now the most common primary glomerular disease causing ESKD in the United States (Kitiyakara et al., 2004; Korbet, 2012). Current therapies for FSGS focus on controlling hypertension and reducing proteinuria by blockade of the renin–angiotensin system (Korbet, 2012). Immunosuppressive therapy is often added based on the rationale that the disease is caused by a dysregulated immune system (D'Agati et al., 2011; Korbet, 2012). Despite these therapies, ~50% of nephrotic patients progress to ESKD over a decade (Korbet, 2012). As a result, there is much interest in developing new therapies.

Glomerular podocytes play a pivotal role in FSGS and other proteinuric kidney diseases (D'Agati et al., 2011; Wiggins, 2007; Wolf et al., 2005). These cells cover the external surface of the glomerular basement membrane and maintain the structural and functional integrity of the glomerular filter (Wiggins, 2007; Wolf et al., 2005). While FSGS is caused by multiple, distinct etiologies (Rosenberg & Kopp, 2017), podocyte injury is a characteristic feature of the disease process, which leads to a decrease in podocyte number (D'Agati et al., 2011; Wiggins, 2007). Because podocytes are terminally differentiated, postmitotic cells, podocytes that are lost cannot be effectively replaced, which causes instability and collapse of the glomerular tuft, glomerulosclerosis, and disease progression (D'Agati et al., 2011; Wiggins, 2007).

Accumulating evidence suggests that cGMP signaling has podocyte protective effects in kidney diseases (Makino et al., 2006; Ogawa et al., 2012; Shen et al., 2016; Staffel et al., 2017; Suganami et al., 2001). The beneficial actions of cGMP are mediated, in part, by inhibiting TGFβ, Rho GTPases, and calcium signaling (Francis et al., 2010; Shen et al., 2016). These signaling pathways promote podocyte loss by inducing detachment and apoptosis (Robins et al., 2017; Wang et al., 2012; Zhu et al., 2011). Thus, inhibition of these signaling pathways by cGMP may attenuate the decrease in podocyte number characteristic of FSGS.

Natriuretic peptides potently stimulate cGMP generation in cultured podocytes (Lewko et al., 2004; Theilig et al., 2001). The family of natriuretic peptides includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (Potter et al., 2006). ANP and BNP are predominantly produced by the heart, and circulate to act on target organs such as the kidney (Potter et al., 2006). CNP is widely expressed in multiple tissues and cell types including the kidney (Cataliotti et al., 2002; Potter et al., 2006); thus, providing a local source of natriuretic peptides.

Natriuretic peptides stimulate cGMP production by binding to cell surface natriuretic peptide receptors (NPRs) (Pandey, 2014; Potter et al., 2006). ANP and BNP bind to NPRA, and CNP binds to NPRB (Pandey, 2014; Potter et al., 2006). In contrast, the natriuretic clearance receptor natriuretic peptide clearance receptor (NPRC) binds and degrades ANP, BNP, and CNP and negatively regulates the effects of natriuretic peptides (Pandey, 2014; Potter et al., 2006). Podocytes express all three NPRs (Lewko et al., 2004; Potter et al., 2006; Staffel et al., 2017). Moreover, NPRC is highly expressed in glomerular podocytes compared to other glomerular and kidney cell types (Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019). Based on these data, we hypothesized that pharmacologic inhibition of NPRC clearance would enhance local natriuretic peptide levels, promote cGMP signaling in podocytes and, in turn, attenuate glomerular injury. To test this hypothesis, we blocked natriuretic peptide clearance using the specific NPRC ligand ANP(4-23) (Anand-Srivastava et al., 1990; Li et al., 2014; Maack et al., 1987; Nishizawa et al., 2017; Veale et al., 2000) We found that NPRC blockade significantly reduced albuminuria in a mouse model of FSGS.

2 | METHODS

2.1 | Materials

Primary antibodies used for the studies included: (1) A mouse monoclonal antibody to α-smooth muscle actin (Durand-Arczynska et al., 1993) (clone 1A4, catalog number: A5228, Sigma-Aldrich), (2) A mouse monoclonal antibody to actin (Lessard, 1988) (clone C4, catalog number: MA1501, Sigma-Aldrich), (3) Goat polyclonal antibodies to neprilysin (Sagare et al., 2013) (catalog number: AF1126) and nephrin (Wong et al., 2018) (catalog number: AF3159), all from R&D Systems (Minneapolis, MN), and (4) A mouse monoclonal antibody to NPRC (clone OTI4H1, catalog number: TA501044, Origene Technologies). Secondary antibodies used for the studies included: (1) A mouse anti-goat polyclonal antibody (catalog number: sc-2354, Santa Cruz Biotechnology), and (2) An anti-mouse polyclonal antibody (catalog number: 7076, Cell Signaling Technology). Additional materials included: ANP(4-23) (GenScript), human ANP, human CNP and PF-04449743 (Sigma-Aldrich), AP-811 (Tocris Bioscience), and LBQ657 and tadalafil (Cayman Chemical).

2.2 | Animal experiments

All experiments were performed using mouse strains on the FVB/NJ background and a transgenic (TG) mouse model of FSGS developed in our laboratory (Wang et al., 2015). These TG mice express a constitutively active Gq α-subunit, which is specifically induced in podocytes with doxycycline (2 mg/ml in drinking water with 2% sucrose to enhance palatability; Wang et al., 2015). In these TG mice treatment with the podocyte toxin puromycin aminonucleoside (PAN) causes robust albuminuria and glomerulosclerosis in TG mice, but only mild disease in non-TG animals.

For the experiments, age and sex-matched male and female mice were used for the studies because the phenotype is similar in both sexes (Wang et al., 2015). Mice were studied at 3–4 months of age and all mice were born within a 5-week period. Baseline 24-h urine collections were collected using metabolic cages specifically designed for collection of mouse urine (Hatteras Instruments). After collecting baseline urines, mice were treated with doxycycline for 1 week and then nephrosis was induced by a single injection of PAN (500 mg/kg) by intraperitoneal (IP) injection and doxycycline was continued an additional 2 weeks. During the 2-week study period, TG and non-TG were treated with the either 10 nmol/kg ANP(4-23) or vehicle (sterile saline) by IP injection 5 days per week (Monday through Friday). Systolic blood pressure was measured as described (Wang et al., 2017) the second week after the PAN injection, following acclimation to the experimental procedure the prior week. Repeat 24-hour urine collections were obtained at day 10 and day 14 following the PAN injection. Mice were euthanized after the last urine collection by injecting 250 mg/kg pentobarbital, IP, followed by bilateral thoracotomy. Blood was immediately obtained by cardiac puncture after euthanasia and then kidneys were removed, weighed and kidney tissue was saved for additional studies as described below. The experiments conformed to the *Guide for the Care and Use of Laboratory Animals* and were approved by the Duke and Durham VA Medical Centers' Institutional Animal Care and Use Committees. A schematic of the experimental protocol is provided in Figure S1.

To assess the effectiveness of ANP(4-23) at enhancing cGMP generation in wild type mice, we performed a pilot study by treating mice with a single injection of ANP(4-23) (10 nmol/kg by IP injection) or vehicle (normal saline). Urines were then collected in metabolic cages, during the day after injection, overnight, and the following morning during the time periods indicated in Figure 2. Data were expressed as nanograms/milligram creatinine.

2.3 | BP measurements

Systolic BP was measured using a computerized tail-cuff system (Hatteras Instruments) in conscious mice as previously described (Wang et al., 2017). To reduce variability in the results, mice were acclimated to the experimental conditions for a week prior to the BP measurements. This technique has previously been shown to correlate closely with intra-arterial measurements (Whitesall et al., 2004).

2.4 | Histopathology

After fixation in formalin, light microscopic sections were stained with hematoxylin and eosin (H&E) and Masson trichrome. The slides were evaluated by a pathologist (A.F.B.) blinded to genotype. Each tissue section had more than 20 glomeruli for evaluation. Tubules were examined for tubule dilation and casts, and tubulointerstitial areas were examined for inflammation with or without interstitial fibrosis. There is little tubulointerstitial fibrosis observed in this model over a 14-day period, and this score was based on the severity of inflammation as described below. Abnormalities were graded using a semi-quantitative scale of 0–3 (0-normal, 1-mild, 2-moderate, 3-severe) as previously described; Wang et al., 2015; Wang et al., 2019) based on the following criteria:

2.4.1 | Glomerulosclerosis

1. Normal (baseline): None.
2. Mild: <10% of glomeruli.
3. Moderate: 10%–25% of glomeruli.
4. Severe: >25% of glomeruli.

2.4.2 | Tubule injury

1. Normal (baseline): None to minimal tubular dilation and casts without tubular degeneration or regeneration.
2. Mild: Tubular degeneration and regeneration with/without tubular dilation and casts, involving <10% of cortex.
3. Moderate: Tubular degeneration and regeneration with/without tubular dilation and casts, involving 10–25% of cortex.
4. Severe: Tubular degeneration and regeneration with/without tubular dilation and casts, involving >25% of cortex.

2.4.3 | Tubulointerstitial inflammation

1. Normal (baseline): One focus of inflammation (up to 15 mononuclear cells) with or without interstitial fibrosis.

2. Mild: Two foci of 15+ mononuclear cells or 1 focus of 30+ mononuclear cells involving up to 5% of area of cortical parenchyma with or without interstitial fibrosis.
3. Moderate: Inflammation involving >5%–25% of the cortical parenchyma with or without interstitial fibrosis.
4. Severe: Inflammation involving >25% of the cortical parenchyma with or without interstitial fibrosis.

2.5 | Cell culture studies

The immortalized mouse podocyte cell line (Schwartz et al., 2001) was a gift of Dr. Paul E. Klotman (Mount Sinai Medical Center, New York City, NY). The cells were derived from animals bred with the H-2Kb-tsA58 Immortomice (Charles River Laboratories). Podocytes were selected for expression of the podocyte markers WT-1, synaptopodin, and podocalyxin. To permit immortalized growth, cells were grown at 33°C in medium supplemented with 100 units/ml gamma-interferon to induce the H-2Kb promoter driving synthesis of the temperature sensitive (tsA58) SV-40 T antigen (permissive conditions). For differentiation, cells were grown at 37°C in medium lacking gamma-interferon, resulting in degradation of the T antigen (non-permissive conditions). Our lab further characterized differentiated cells by RT-PCR and they also express low levels of the podocyte proteins nephrin and podocin. For the experiments, the immortalized mouse podocyte cell line was maintained in culture and were plated in either 6- or 12-well tissue culture clusters (Corning-Costar) and differentiated for ~7 days prior to study as previously described (Flannery & Spurney, 2006).

For the cGMP studies, podocytes were made quiescent by incubation in serum free medium overnight. Cells were then treated with vehicle or the following concentrations of compounds in the presence of the following compounds: 5 μM PF-04449743, 5 μM tadalafil, 10 μM LBQ657, 1 μM ANP(4-23), 100 μM SNAP, 1 μM CNP, and either 0.1 nM, 1 nM, 10 nM, or 1 μM ANP as indicated. After 30 min, the supernatant was aspirated, and the tissue culture clusters placed on ice. Hydrochloric acid (0.1 N) was then added to the wells (100 μl per 12 wells and 300 μl per 6 well tissue culture cluster). A cell scrapper was used to remove the cells from the cell culture wells, and the mixture dissociated by pipetting up and down until homogenous. The mixture was then centrifuged at 1000 *g* for 10 min and the supernatant frozen at −80°C. Measurement of cGMP in the supernatants was performed using an ELISA kit from Cayman Chemicals according to the directions of the manufacturer.

For the apoptosis studies, cells were changed to medium with 0.1% fetal bovine serum (FBS) overnight and the following morning cells were treated with either vehicle, 50 μM PAN, 1 μM ANP, and/or 1 μM CNP as indicated. Additional ANP or CNP was added to the medium in the afternoon prior to study to supplement any ANP which may have been degraded during the incubation period. The following morning, cells were harvested and apoptotic cells were identified by annexin V staining using kits from BD Pharmingen according to the directions of the manufacturer. Quantitation of the apoptotic cells was performed by flow cytometric analysis at the Duke Comprehensive Cancer facility. For the annexin V studies, apoptotic podocytes were differentiated from necrotic cells by staining with 7-amino-actinomycin D. For evaluation of these data, basal levels of apoptosis (cells treated with 0.1% FBS and vehicle) were subtracted from the results, and data expressed as the percent apoptosis above basal.

2.6 | Expression of glomerular mRNAs

Reverse transcription (RT) followed by a quantitative polymerase chain reaction (Q-RT-PCR) was performed using an iCycler TM (Bio-Rad Laboratories, Inc.). For the studies, total cellular RNA was prepared using glomerular preparations and the Trizol reagent according to the manufacturer's directions (Life Technologies Inc.). The RNA was treated with RNAase free DNAase (Qiagen) and then reverse-transcribed with Superscript reverse transcriptase (Invitrogen) and oligo (dT) primers. Real-time quantitative PCR was performed using an iCycler Q-PCR machine and the universal SYBR Green PCR Master Mix Kit (Perkin-Elmer). The amplification signals were normalized to the endogenous cyclophilin mRNA level. The primer sequences used for Q-RT-PCR were as follows: CNP forward: 5′-AAT ACA AAG GCG GCA ACA AG—3′ & reverse, 5′—TAA CAT CCC AGA CCG CTC AT—3′. NPRC forward, 5′—AGC TGG CTA CAG CAA GAA GG—3′ & reverse, 5′—CGG CGA TAC CTT CAA ATG TC—3′, Neprilysin forward, 5′—CCA AAC TTA AGC CTA TTC TTA C—3′ & reverse, 5′—CCA TTA TGA ACC TCC AGG AC—3′, PDE5a forward, 5′—AGA CAT GG TCA ACG CAT GGT T—3′ & reverse, 5′—TAT GGG CTC GGA TGC CTT C—3′, PDE9a forward, 5′—ACC TGT TCT GTA TCG CCA CC—3′ & reverse, 5′—CTT CAC AGC CAC AGG TCT CA—3′, Cyclophilin, forward, 5′—GGC CGA TGA CGA GCC C—3′ & reverse, 5′—TGT CTT TGG AAC TTT GTC TGC AA—3; Fibronectin forward 5′—CGA GGT GAC AGA GAC CAC AA—3′ & reverse 5′—CTG GAG TCA AGC CAG ACA CA—3′; collagen type 1, α1 (COL1A1) forward 5′—ATC TCC TGG TGC TGA TGG AC—3′ & reverse 5′—ACC TTG TTT GCC AGG TTC AC—3′; α-smooth muscle actin (SMA) forward 5′—GAG GCA CCA CTG ACC CCT AA—3′ & reverse 5′—CAT CTC CAG AGT CCA GCA CA—3′.

2.7 | Statistical analysis

Data are presented as the mean ± standard error of the mean and statistical analyses were performed using the Prism computer program (GraphPad Software, Inc.). For comparison of two groups of continuous variables, data were analyzed by a *t*-test. For comparison of more than 2 groups of continuous variables, data were analyzed by either: (1) A one-way or two-way analysis of variance (ANOVA) as specified by the Prism program, followed by Sidak's multiple comparisons post-test. (2) A Kruskal–Wallis test followed by Dunn's multiple comparison post-test, or (3) An ANOVA followed by Dunnett's multiple comparisons post-test. For non-continuous variables (histopathology), data were analyzed using a Fishers exact test using the number mice with the specified histologic abnormality. Graphs of the histologic findings were presented as the percentage of mice with the specified abnormality to permit a more effective comparison of the differences between the experimental groups in studies with an imbalance in the number of mice in each group.

3 | RESULTS

3.1 | Effect of natriuretic peptides on podocyte cGMP generation and apoptosis

Generation of cGMP is stimulated by nitric oxide and by natriuretic peptides (Francis et al., 2010). Natriuretic peptides potently stimulate cGMP generation in cultured podocytes; whereas, the effects of nitric oxide on podocyte cGMP generation are of a lesser magnitude (Lewko et al., 2004, 2006; Theilig et al., 2001). As shown in Figure 1a, we measured podocyte cGMP generation in the presence or absence of ANP, CNP, and the nitric oxide donor SNAP (S-nitroso-*N*-acetyl-DL-penicillamine). Both ANP and CNP potently stimulated cGMP generation in cultured podocytes. In contrast, stimulation of podocytes with SNAP-induced little cGMP generation. We next determined if ANP protected glomerular podocytes from apoptosis induced by PAN. As shown in Figure 1b, treatment with PAN increased apoptosis above basal levels, and this increase in apoptosis was significantly inhibited by treatment with either ANP or CNP. Taken together with published studies (Lewko et al., 2006; Staffel et al., 2017; Theilig et al., 2001), these data suggest that: (1) ANP and CNP potently stimulate cGMP generation in podocytes, (2) ANP and CNP have podocyte protective effects in vitro, and (3) Given that ANP stimulates NPRA and CNP stimulates NPRB, podocytes express both NPRA and NPRB.

In addition to expressing NPRA and NPRB, podocytes express high levels of the NPRC (Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019), which binds and degrades natriuretic peptides (Potter et al., 2006). Natriuretic peptide-induced cGMP generation is also negatively regulated by additional mechanisms including phosphodiesterases (PDEs) and the neutral endopeptidase (neprilysin) (Potter et al., 2006). The family of PDE isoenzymes hydrolyze both cAMP and/or cGMP within the cell, and these enzymes are found in multiple tissues and cell types (Dousa, 1999; Maurice et al., 2014). However, only PDE5 and PDE9 are selective for cGMP, and are expressed in kidney (Dousa, 1999; Maurice et al., 2014). Neprilysin cleaves and inactivates circulating natriuretic peptides and the enzyme is also widely expressed (Potter et al., 2006). Moreover, high levels of the enzyme are expressed in podocytes (Debiec et al., 2002; Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019). As shown in Figure 1c,d, mRNA for NPRC, neprilysin, PDE5, and PDE9 are expressed in cultured podocytes and both NPRC and neprilysin are highly expressed at both the mRNA and protein level. Thus, multiple mechanisms likely regulate natriuretic peptide responsiveness in glomerular podocytes.

To examine the most effective strategy to enhance natriuretic peptide-induced cGMP generation in podocytes, we measured ANP-induced cGMP generation in the presence of the ANP analog ANP(4-23), which specifically blocks binding of natriuretic peptides to NPRC without affecting NP binding to either NPRA or NPRB (Maack et al., 1987; Nishizawa et al., 2017; Veale et al., 2000; William et al., 2008). We compared the effects of ANP(4-23) with the neprilysin inhibitor LBQ657 (Campbell, 2017), the specific PDE5 inhibitor tadalafil (Rotella, 2002) the specific PDE9 inhibitor PF-04449613 (Lee et al., 2015) at doses that effectively blocked either NPRC, neprilysin, PDE5, or PDE9. As shown in Figure 1e, blockade of NPRC-enhanced cGMP generation at low nanomolar concentrations of ANP; whereas neprilysin inhibition and inhibition of either PDE5 or PDE9 had little effect on cGMP generation at the concentrations of ANP used in the experiments.

3.2 | Effect of ANP (4-23) on urinary cGMP generation in wild type mice

Based on these findings in cultured podocytes, we hypothesized that augmenting natriuretic peptide signaling by blockade of NPRC might enhance the podocyte protective actions of endogenous natriuretic peptides in vivo. To investigate this possibility, we evaluated the effect of ANP (4-23) in wild type mice. For the studies, urinary cGMP excretion was measured after a single, IP injection of ANP (4-23) as shown in Figure 2. Urine was collected at the indicated times over a 24-hour period (Figure 2a, period 1, 2, and 3). There was a significant increase in urinary

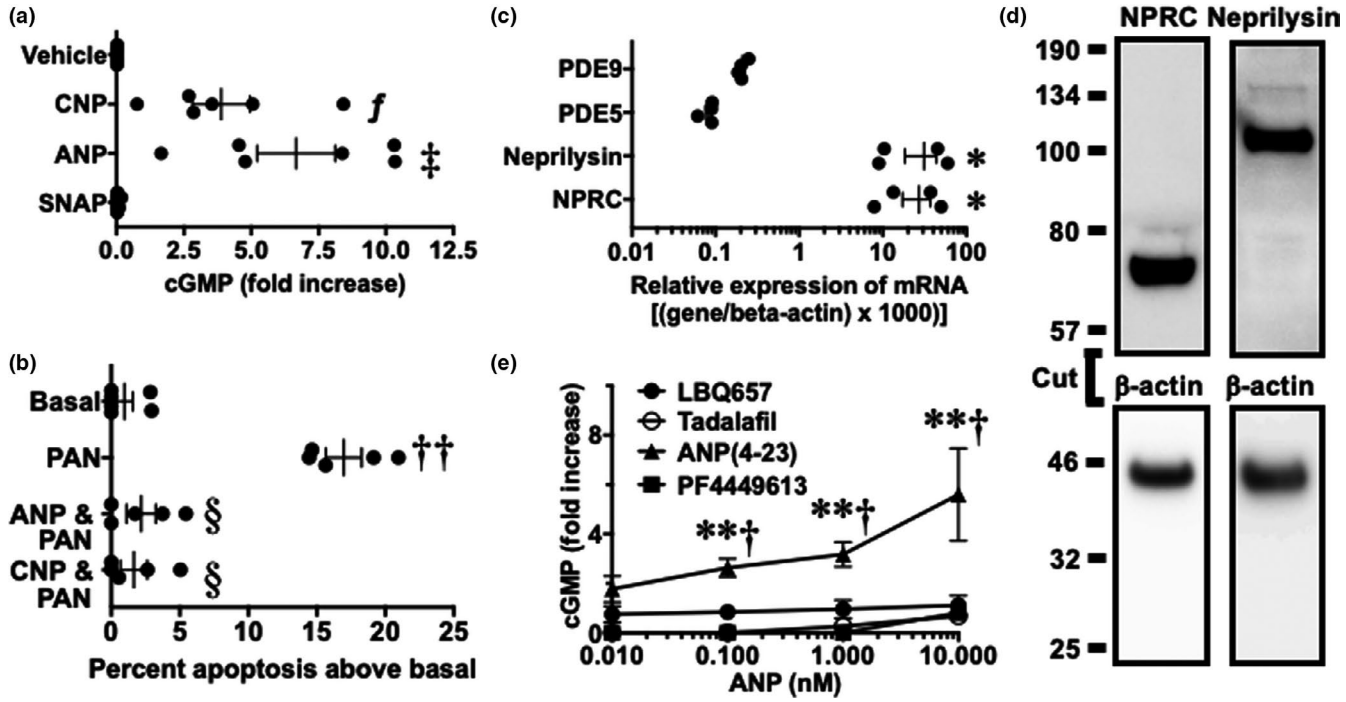

FIGURE 1 Effect of natriuretic peptides on podocyte cGMP generation and apoptosis. (A) Both ANP (1 μM) and CNP (1 μM) potently stimulated cGMP generation. In contrast, SNAP (100 μM)-induced little cGMP generation. (b) PAN significantly enhanced apoptosis and this increase in apoptosis was inhibited by ANP and CNP. (c & d) Podocyte express mRNA for NPRC, neprilysin, PDE5, and PDE9. NPRC and neprilysin were highly expressed in cultured podocytes at both the mRNA and protein level. (e) Podocytes were treated with the indicated doses of ANP in the presence or absence of the NPRC ligand ANP(4-23) (1 μM), the neprilysin inhibitor LBQ657 (10 μM), the PDE5 inhibitor tadalafil (5 μM) or the PDE9 inhibitor PF-04449613 (5 μM). Pharmacologic blockade of NPRC was the most effective strategy to potentiate natriuretic peptide-induced cGMP generation. Four to six tissue culture wells were studied in each group. $^{f}p < 0.01$ or $^{‡}p < 0.001$ versus vehicle by ANOVA followed by Sidak's multiple comparisons post-test, $^{*}p < 0.05$ versus PDE5 by Kruskal–Wallis test followed by Dunn's multiple comparisons test, $^{§}p < 0.01$ versus PAN or $^{††}p < 0.001$ versus basal by an ANOVA followed by Sidak's multiple comparisons post-test, $^{**}p < 0.01$ versus LBQ657 or $^{†}p < 0.001$ versus tadalafil, or PF4449613 by ANOVA followed by Sidak's multiple comparisons post-test

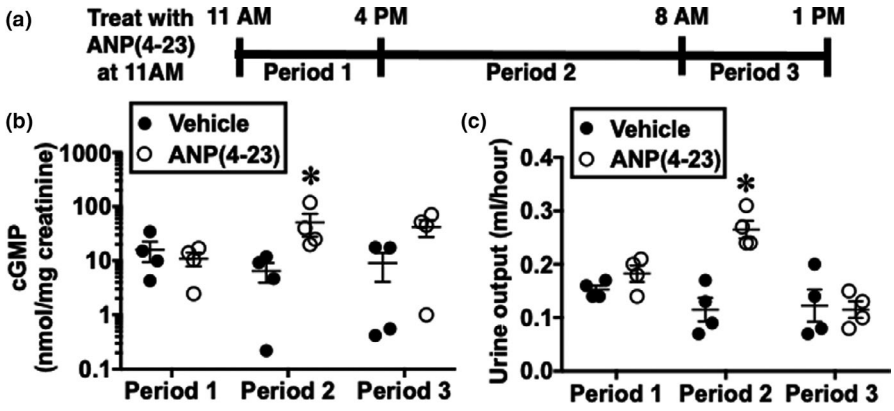

FIGURE 2 Effect of ANP(4-23) on urinary cGMP excretion in non-TG wild type mice. (a) Mice were given a single, IP injection of 10 nmol/kg ANP(4-23) or vehicle at the beginning of period 1 (11AM). Urine was then collected during periods 1, 2, and 3 at the indicated times. (b) Urinary cGMP excretion was significantly increased in period 2, and the increase in urinary cGMP excretion was sustained in period 3. (c) Urine output increased significantly during period 2 and returned to baseline in period 3. Four non-TG mice were studied in each group. $^{*}p < 0.05$ versus vehicle by ANOVA followed by Dunnett's multiple comparisons post-test. Data in (c) were divided by time (h) to correct for the different durations of the urine collections. Note that the *y*-axis of (a) is logarithmic

cGMP excretion in wild type mice during the overnight collection that was sustained in the urine collected the following day (Figure 2b). Moreover, urine output increased significantly during the nighttime urine collection (Figure 2c).

3.3 | Effect of ANP(4-23) on kidney disease in TG mice

We next evaluated the effects of ANP(4-23) on kidney disease in TG mice (Wang et al., 2015) using the protocol described in Section 2 (see also the schematic in Figure S1). This TG model expresses a doxycycline inducible, constitutively active Gq α-subunit specifically in podocytes (Wang et al., 2015). In these animals, treatment with a single dose of the podocyte toxin PAN causes robust albuminuria in TG mice, but only mild disease in non-TG animals. For the experiments, we chose a 10 nmol/kg dosage of ANP(4-23) that had previously been demonstrated to reduce systemic blood pressure in spontaneously hypertensive rats but not in control Wistar-Kyoto rats (Li et al., 2014). Figure 3 shows the effects of ANP(4-23) on albuminuria, glomerular histology, and systemic blood pressure. We found that PAN-induced heavy proteinuria at day 14 in TG mice treated with vehicle (normal saline) compared to baseline albuminuria (Figure 3a). This increase in albuminuria at day 14 was significantly reduced by treatment with ANP(4-23) (Figure 3a). PAN had little effect on albuminuria in non-TG mice (Table 1). Treatment with ANP(4-23) also reduced glomerular injury (Figure 3b–f), but this difference was not statistically significant. A similar pattern was seen in tubulointerstitial inflammation and tubular dilation and casts (Table 2). Systolic BP was similar in TG mice receiving ANP(4-23) and in the vehicle-treated group (Figure 3g). There was no clear relationship between the severity of glomerular injury and systolic BP (Figure S2). A subgroup analysis of mice with severe glomerulosclerosis found a systolic BP of 127.5 ± 2.3 mmHg ($N = 4$) in the TG mice treated with vehicle compared to a systolic BP of 132 ± 5.7 mmHg ($N = 5$; p = NS) in the ANP(4-23)-treated TG mice.

3.4 | Effect of male and female sex on the experimental outcomes

To examine the sex-dependent effects of the treatment, albuminuria, and glomerular histologic findings are presented for male and female mice in Figure 4. As shown

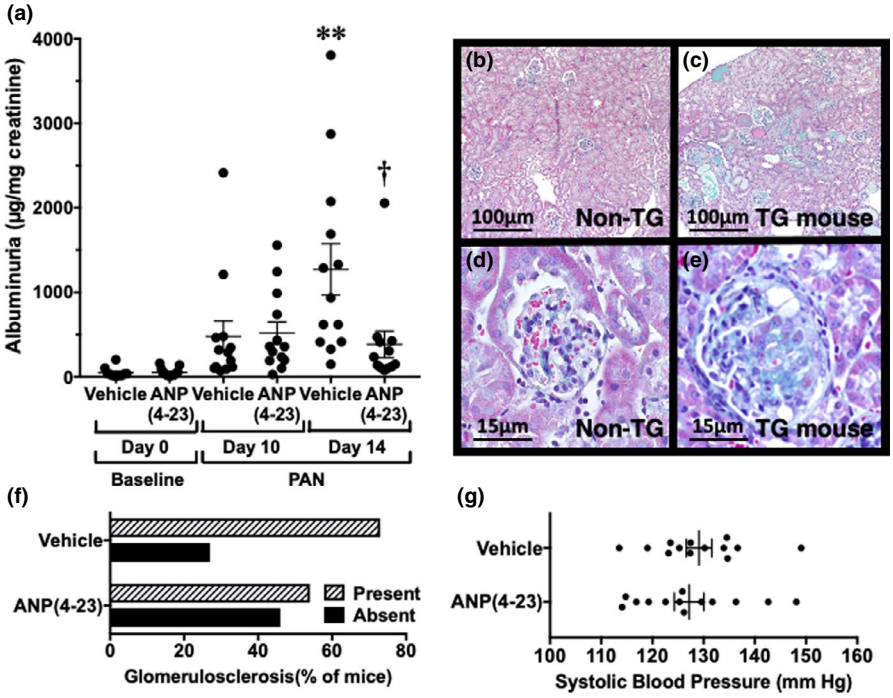

FIGURE 3 Effect of ANP(4-23) on kidney disease in TG mice. (a) PAN-induced heavy proteinuria at day 14 in TG mice treated with vehicle compared to baseline albuminuria. The increase in albuminuria at day 14 was significantly reduced by treatment with ANP(4-23). (b–f) Treatment with ANP(4-23) reduced the number of mice with glomerular injury but this difference was not statistically significant. (g) Systolic BP was similar in TG mice treated with vehicle and ANP(4-23). Ten to 13 TG mice were studied in each group. $^{**}p = 0.0002$ versus baseline or $^{\dagger}p = 0.003$ versus vehicle-treated TG mice at day 14 by ANOVA followed by Sidak's multiple comparisons post-test. Kidney sections were stained with Masson's trichrome stain

in Figure 4a, albuminuria was significantly increased in female mice on day 14 and this increase in albuminuria was significantly reduced by treatment with ANP (4-23). A similar pattern was seen in male mice, but the differences were not statistically significant. There was a significant effect of treatment with ANP(4-23) on albuminuria ($p = 0.0016$) by a two-way analysis of variance (ANOVA), but there was no significant effect of sex on the results. Consistent with the albuminuria, the severity of glomerulosclerosis was more severe in female mice compared to male animals in both the vehicle-treated group (Figure 3b) and the ANP-(4-23) treated group (Figure 4c), but these differences were not statistically significant. Lastly, there was no significant difference in glomerulosclerosis by sex in mice treated with ANP(4-23) compared to mice treated with vehicle (compare Figure 4b,c).

We next evaluated the effect of ANP(4-23) treatment on expression of podocyte protein mRNAs (nephrin, podocin, Wilms tumor 1, or WT-1), markers of fibrosis (fibronectin, collagen type 1, α1, or COL1A1) and a marker myofibroblast differentiation (α-smooth muscle actin or α-SMA) (Davis et al., 2012; Tomasek et al., 2002). In these studies, mRNA levels of these transcripts were similar in the ANP(4-23)- and vehicle-treated non-TG groups and these data were combined for the analyses. As shown in Table 3, there was no significant differences in expression of mRNAs for nephrin, podocin, and WT-1 in both TG groups compared to non-TG control mice. In contrast, markers of fibrosis and myofibroblast differentiation were increased in both groups of TG mice compared to non-TG controls, and these differences were statistically significant for fibronectin and COL1A1.

TABLE 1 Albuminuria (μg/mg creatinine) in non-TG controls

	Vehicle	ANP(4-23)
Baseline	11.8 ± 0.8	13.7 ± 1.7
10 days	9.9 ± 1.5	12.8 ± 1.7
14 days	16.9 ± 4.0	9.0 ± 1.4

Note: Data are expressed as the mean ± SEM. Number of mice: Vehicle = 6, ANP(4-23) = 7.

3.5 | Effect of ANP(4-23) on glomerular podocytes and kidney fibrosis

Figure 5a–c show the effects of ANP(4-23) on expression of nephrin and α-SMA proteins in mouse glomerular preparations. As shown in Figure 5a,b, nephrin levels were better preserved in ANP(4-23)-treated TG mice compared to TG mice treated with vehicle. Consistent with the mRNA studies (Table 3), α-SMA protein levels were significantly increased in glomerular preparations from TG mice treated with either vehicle or ANP(4-23) (Figure 4a,c). A similar pattern was observed in collagen expression in kidney cortex by Sirius red staining in TG mice compared to non-TG mice (Figure 5d) but this difference was not significant.

TABLE 2 Histopathologic findings (number of mice)

	Glomerulosclerosis	Tubular dilation & casts	Tubulointerstitial inflammation
TG vehicle ($N = 11$)	None 3	None 2	None 2
	Mild 2	Mild 6	Mild 5
	Moderate 2	Moderate 3	Moderate 4
	Severe 4	Severe 0	Severe 0
TG ANP(4-23) ($N = 11$)	None 5	None 3	None 4
	Mild 1	Mild 7	Mild 2
	Moderate 0	Moderate 1	Moderate 5
	Severe 5	Severe 0	Severe 0
Control Vehicle ($N = 5$)	None 6	None 6	None 6
	Mild 0	Mild 0	Mild 0
	Moderate 0	Moderate 0	Moderate 0
	Severe 0	Severe 0	Severe 0
Control ANP(4-23) ($N = 5$)	None 6	None 6	None 6
	Mild 0	Mild 0	Mild 0
	Moderate 0	Moderate 0	Moderate 0
	Severe 0	Severe 0	Severe 0

Note: During the study, two female TG mice died in the vehicle group and two TG mice (1 male and 1 female) died in the ANP(4-23) group.

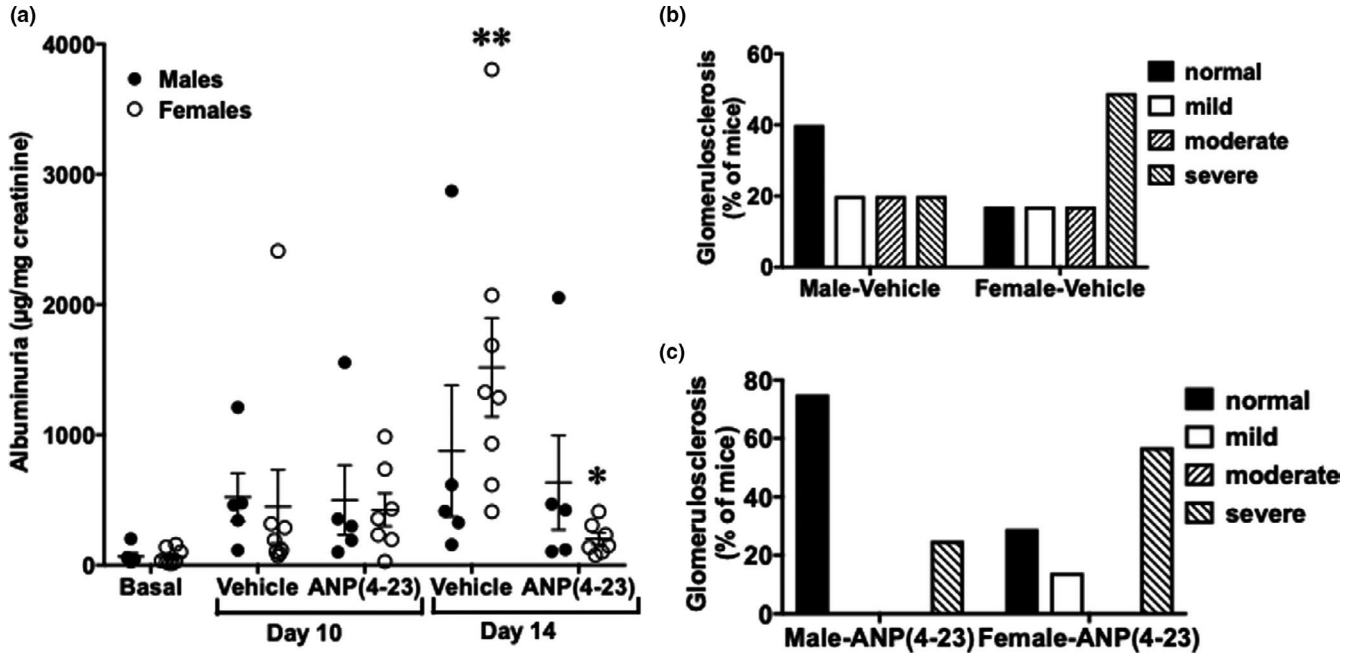

FIGURE 4 Effect of sex on the experimental outcomes. (a) Albuminuria was significantly increased in female mice on day 14 and this increase in albuminuria was significantly reduced by treatment with ANP(4-23). By a two-way ANOVA, there was a significant effect of treatment with ANP(4-23) on albuminuria but no significant effect of male or female sex on the experimental outcome. (b and c) There was no significant difference in glomerulosclerosis in male mice compared to female mice in both the vehicle and ANP(4-23)-treated groups. In addition, there was no significant difference in glomerulosclerosis by sex between mice treated with ANP(4-23) versus mice treated with vehicle (compare panels b & c). *$p \leq 0.0126$ versus vehicle-treated female mice or **$p = 0.0062$ versus basal levels in female mice by two-way ANOVA followed by Sidak's multiple comparisons post-test

TABLE 3 Relative expression of mRNA (mean ± SEM)

	Non-TG Controls	TG mice Vehicle	TG mice ANP(4-23)
Nephrin	1.0000 ± 0.5782	0.7501 ± 0.2819	0.7964 ± 0.1905
Podocin	1.0000 ± 0.5880	0.7355 ± 0.6438	0.7683 ± 0.1992
WT-1	1.0000 ± 0.3989	1.0630 ± 0.2990	0.9896 ± 0.1789
α-SMA	1.0000 ± 0.3356	2.877 ± 0.6527	2.143 ± 0.5401
Fibronectin	1.0000 ± 0.4904	11.49 ± 4.0060**	10.59 ± 2.425*
COL1A1	1.0000 ± 0.5782	18.44 ± 7.038†	14.18 ± 6.893**

Note: Data are expressed as the mean ± SEM analyzed by a Kruskal–Wallis test followed by Dunn's multiple comparison test ($^{\dagger}p < 0.005$, **$p < 0.01$, or *$p < 0.025$ vs. non-TG controls). Transcript levels were similar in the ANP(4-23)- and vehicle-treated non-TG groups and these data were combined for the analyses.

There were no significant differences in podocyte numbers in the TG and non-TG animals (Figure 5e).

3.6 | Effect of ANP(4-23) on urinary cGMP excretion in TG and non-TG mice

Lastly, we measured urinary cGMP excretion to assess the effectiveness of NPRC blockade. There was no significant difference in urinary cGMP excretion in TG mice at baseline. Treatment with PAN-enhanced urinary cGMP excretion in all groups of mice compared to the baseline measurements. This increase in urinary cGMP excretion was statistically significant compared to baseline in TG mice treated with either vehicle or ANP(4-23) (Figure 6). There was also an increase urinary cGMP excretion in ANP(4-23)-treated TG mice compared to the vehicle-treated TG animals, but this difference was not statistically significant after correcting for multiple comparisons.

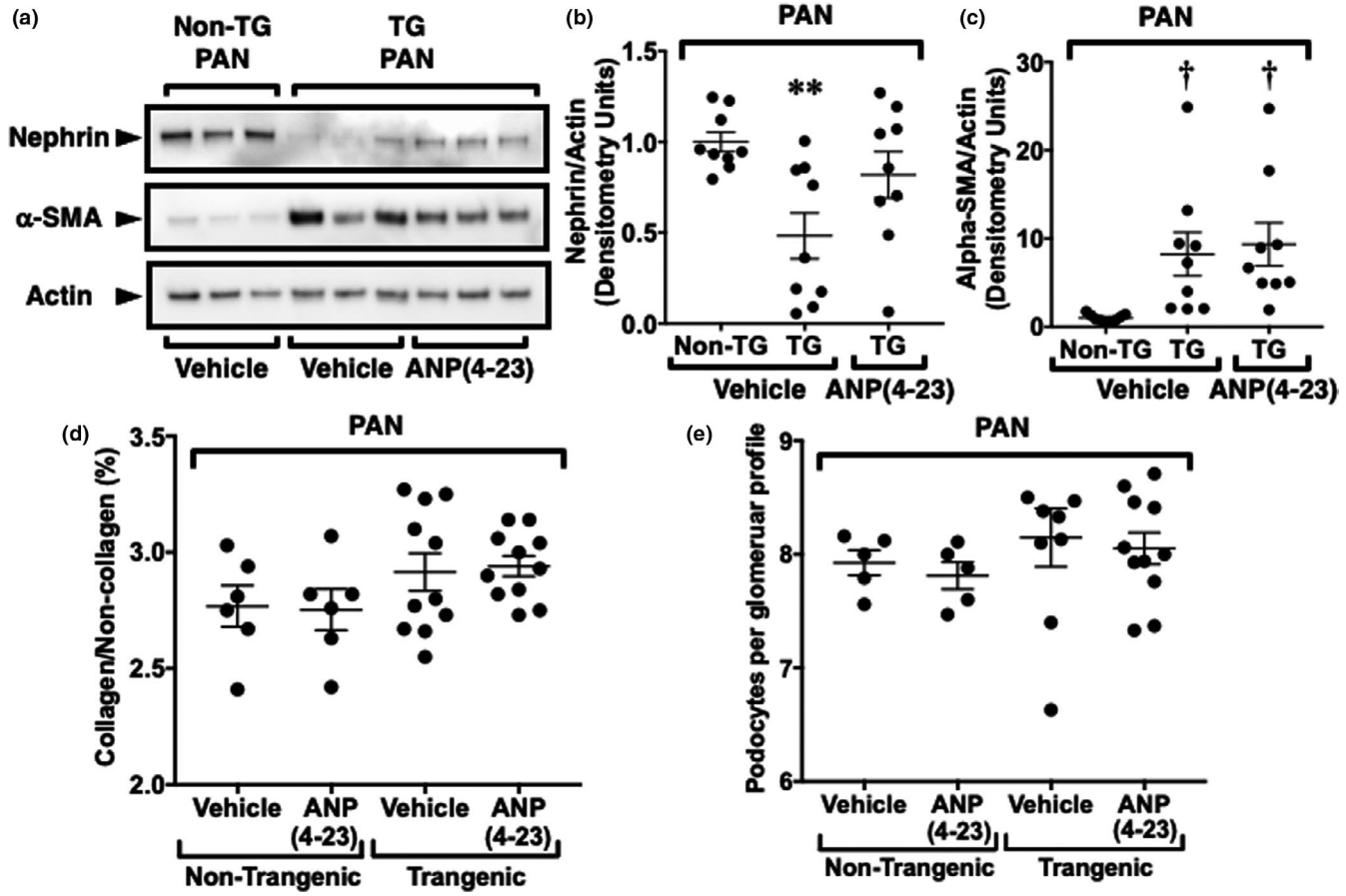

FIGURE 5 Effect of ANP(4-23) on glomerular podocytes and kidney fibrosis. (a & b) Expression of nephrin was significantly reduced in the vehicle-treated TG mice compared to non-TG mice treated with PAN. ANP(4-23) significantly inhibited this decrease in nephrin expression. (a & c) Expression of α-SMA was similarly increased in both groups of TG mice compared to non-TG controls treated with PAN. (d) A similar nonsignificant increase in fibrosis was observed in the TG mice compared to the non-TG mice by Sirius red staining in kidney cortex. (e) There were no significant differences in podocytes per glomerular profile in the TG and non-TG groups. Nine mice were studied in each group for the immunoblotting studies. $^{**}p < 0.01$ versus non-TG mice by an ANOVA followed by Sidak's multiple comparisons post-test; $^{\dagger}p < 0.001$ versus non-TG mice by a Kruskal–Wallis test followed by Dunn's multiple comparison post-test

4 | DISCUSSION

In this study, we found that: (1) Both ANP and CNP protected cultured podocytes from PAN-induced apoptosis. (2) Pharmacologic blockade of NPRC was more effective at enhancing podocyte cGMP generation compared to either neprilysin inhibition or inhibition of either PDE5 or PDE9. (3) Blockade of NPRC in vivo inhibited albuminuria and improved expression of the podocyte marker nephrin in a mouse model of FSGS. (4) The beneficial effects of pharmacologic NPRC blockade were accomplished without a significant change in systemic blood pressure, and (5) There was a trend toward improvement in the histopathologic features of the disease but markers of glomerular fibrosis and myofibroblast differentiation were not significantly affected by the treatment strategy. Treatment with ANP(4-23) increased urinary cGMP excretion in TG mice compared vehicle-treated TG mice, but this difference was not statistically significant. Given that ANP(4-23) is rapidly degraded in mouse serum (Nishizawa et al., 2017), we posit that more effective pharmacologic inhibition of NP clearance may further enhance the beneficial effects of the treatment strategy.

Multiple mechanisms negatively regulate the biological effects of natriuretic peptides including NPRC, neprilysin, and PDEs such as the cGMP-specific PDEs including PDE5 and PDE9 (Potter et al., 2006; Rotella, 2002). All these mechanisms likely play a role in regulating the biological effects of natriuretic peptides in glomerular podocytes. Both NPRC and neprilysin compete for extracellular NP ligands; whereas, PDEs act intracellularly to hydrolyze ATP and/or cGMP. Cultured podocytes expressed high levels of both NPRC and neprilysin, consistent with published studied (Debiec et al., 2002; Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019). Moreover, both NPRC and neprilysin play important roles in removing natriuretic peptides from the circulation and inhibiting their activity (Potter et al., 2006). Blockade of NPRC was, however,

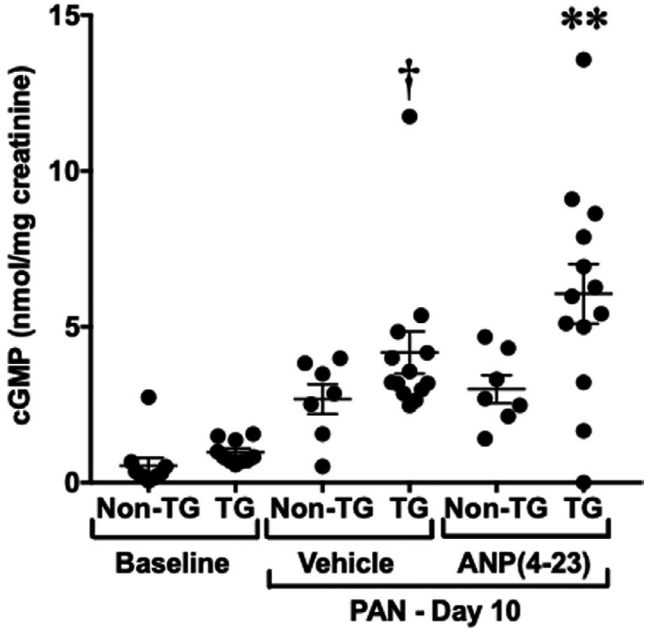

FIGURE 6 Effect of ANP(4-23) on urinary cGMP excretion in TG and non-TG mice. There were no significant differences in urinary cGMP excretion at baseline. Treatment with PAN increased urinary cGMP excretion, and this increase in urinary cGMP excretion from baseline was statistically significant in TG mice treated with either vehicle or ANP(4-23). There was no significant difference in urinary cGMP excretion between TG mice treated with PAN and vehicle compared to TG mice treated with PAN and ANP(4-23). Seven to 10 mice were studied in each group. $^{\dagger}p < 0.01$ or $^{**}p < 0.001$ versus baseline in TG mice by an ANOVA followed by Sidak's multiple comparisons post-test

the most effective approach for augmenting ANP-induced cGMP generation in cultured podocytes compared to inhibition of either neprilysin, PDE5, or PDE9 inhibition (Figure 1e). This finding is consistent with published studies using pharmacologic inhibitors of NPRC or neprilysin in vivo (Potter, 2011). As a result, infusion of NPRC antagonists enhances both ANP concentrations and the physiologic effects of natriuretic peptides to a greater extent than pharmacologic neprilysin inhibition (Charles et al., 1996; Hashimoto et al., 1994; Kukkonen et al., 1992; Okolicany et al., 1992; Potter, 2011). The potent ability of NPRC blockade to enhance cGMP generation in cultured podocytes may be due to: (1) PDEs hydrolyze cGMP inside the cell and inhibition is effective only if significant intracellular cGMP is generated, (2) The high levels of NPRC expressed by podocytes (Debiec et al., 2002; Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019), (3) The high affinity of NPRC for natriuretic peptides (10–140 pM) compared to the lower affinity of neprilysin (Km ~10 to 100 μM) (Hubers & Brown, 2016; Potter et al., 2006). Given the low concentrations of natriuretic peptides in biological fluids (1–100 pM) (Potter et al., 2006), NPRC is more effective than neprilysin at scavenging extracellular natriuretic peptides. These differences are an important advantage of NPRC blockade compared to other pharmacologic approaches for enhancing cGMP generation in podocytes.

ANP (4-23) is a specific NPRC ligand with low nanomolar affinity for NPRC (Anand-Srivastava et al., 1990; Li et al., 2014; Maack et al., 1987; Nishizawa et al., 2017; Veale et al., 2000). In these studies, we found that a single, 10 nmol/kg dose of ANP(4-23) (~1.1 mg/kg) significantly enhanced urinary cGMP excretion in non-TG wild type mice (Figure 2). These data suggest that treatment with ANP(4-23) effectively inhibited NP clearance in wild type mice. We did not anticipate the increase in urinary cGMP excretion in all groups of mice treated with PAN (Figure 5). This enhanced cGMP excretion may have hindered detecting a difference in urinary cGMP excretion in TG mice treated with ANP(4-23) compared to vehicle-treated TG mice by adding another variable that affected cGMP generation in vivo and, in turn, increased variability in the data. Alternatively, repeated dosages of the drug may have reduced the effectiveness of the treatment approach (Agvald et al., 1999; Freedman & Lefkowitz,). This desensitization to pharmacologic therapy is characteristic of agonist drug therapy (Agvald et al., 1999; Freedman & Lefkowitz,), which is directly relevant to cGMP signaling (Agvald et al., 1999). In addition to inhibiting NPRC clearance, ANP(4-23) is reported to stimulate intracellular signaling by NPRC including activating phospholipase C, inhibiting cAMP generation via Gi, and inhibiting L-type ion channels (PLC) (El Andalousi et al., 2013; Li et al., 2014; Murthy et al., 1998; Rose & Giles, 2008; William et al., 2008). Thus, the effects of ANP(4-23) might be mediated, at least in part, by stimulating NPRC. Future studies could address this possibility by studying other high affinity, specific NPRC ligands such as AP-811 (Koyama et al., 1994; Nishizawa et al., 2017; Veale et al., 2000; William et al., 2008) which does not act as an agonist at the NPRC receptor (Becker et al., 2014; William et al., 2008) but effectively inhibits NP clearance from the circulation in vivo (Wegner et al., 1995).

The studies used both male and female mice for the experiments because previous studies found no significant difference in the outcomes by sex (Wang et al., 2015). In the present study, we also found no significant difference in albuminuria or glomerular injury in groups of male and female mice treated with either vehicle or ANP(4-23). However, the improvement in albuminuria was predominantly the result of a decrease in albuminuria in the female animals (see Figure 4). While there was a significant effect of treatment [vehicle versus ANP(4-23)] on the experimental outcomes, sex had no significant effect on either albuminuria or glomerulosclerosis using a two-way ANOVA. It is possible that female mice are more

responsive to the benefits of NPRC blockade compared male mice; however, this possibility will require further study.

In addition to reducing albuminuria in the TG animals, pharmacologic blockade of NPRC improved expression of the podocyte protein nephrin in ANP(4-23)-treated mice compared to TG mice treated with vehicle. This beneficial effect occurred without significantly affecting nephrin mRNA levels in podocytes. Regulation of nephrin expression is complex and includes both transcriptional and post-transcriptional mechanisms (Ren et al., 2005; Ristola et al., 2013; Yamauchi et al., 2006). While we can only speculate on the molecular mechanism(s), nephrin plays a key role in maintaining glomerular filtration barrier integrity (Li et al., 2015). Thus, the beneficial effects of NPRC blockade on podocyte nephrin levels may have contributed to the decrease in albuminuria observed in this study.

In contrast to the beneficial effects of pharmacologic NPRC blockade on albuminuria and nephrin expression, ANP(4-23) had little effect on markers of fibrosis or myofibroblast differentiation. The lack of a beneficial effect on these experimental outcomes may be due to an insufficient drug dosage or desensitization to the effects of the drug as mentioned above. In addition, a subgroup analysis of mice with severe glomerulosclerosis found a trend toward increased systolic BP in ANP(4-23)-treated TG mice compared to vehicle-treated TG mice, which could enhance fibrosis in the ANP(4-23) group. Alternately, increasing the duration of the study may be required to observe a beneficial effect of drug treatment on markers of fibrosis or myofibroblast differentiation. Lastly, pharmacologic potentiation cGMP generation may have effects on renal hemodynamics (Tapia et al., 2012), which may have contributed to the decrease in albuminuria, without affecting glomerulosclerosis.

Lastly, we found that stimulation of either the NPRA or NPRB receptor potently stimulated cGMP generation in cultured podocytes, consistent with a published study (Lewko et al., 2004). In contrast to this previous study (Lewko et al., 2004), however, we found that ANP was a more potent agonist than CNP at similar concentrations (1 μM). While the differing findings may be related to the specific cell lines and/or culture conditions used in the experiments, previous studies suggest: (1) NPRA is highly expressed in glomerular podocytes compared to more modest expression of NPRB (Park et al., 2018), and (2) Activation of NPRB by CNP generally induces cGMP generation of a lower magnitude than activation of NPRA by ANP (Pandey, 2014). These findings may explain the more potent effects on cGMP generation of ANP compared to CNP in the present study.

In summary, NPs protect podocytes from apoptosis. Inhibition of NP clearance is a potent strategy to stimulate NP-induced cGMP generation in both cultured podocytes and in vivo. In a mouse model of FSGS, pharmacologic NPRC blockade decreases albuminuria and preserves podocyte nephrin expression but did not significantly affect the histopathologic features of the disease, systemic blood pressure, or markers of renal fibrosis and myofibroblast differentiation. These data suggest that pharmacologic blockade of NPRC is a promising strategy to treat glomerular diseases. Optimizing the effectiveness of NPRC blockade may further enhance the beneficial effects of this treatment approach.

ACKNOWLEDGMENTS

The results presented in this paper have not been published previously, in whole or in part, except in abstract format. Author contributions to the studies are: (1) Yuping Tang bred the mice for the experiments and collected the blood, tissue, and urine specimens; (2) Liming Wang MD, PhD, analyzed the urine, blood, and tissue samples and contributed to the statistical analyses and preparation of the manuscript. (3) Anne Buckley, MD, PhD, evaluated the renal histology, and (4) Robert Spurney, MD, was involved in all aspects of the design and organization of the research project, and contributed to manuscript preparation. The authors have no conflict of interest to disclose.

ORCID

Robert F. Spurney https://orcid.org/0000-0002-3858-0317

REFERENCES

Agvald, P., Adding, L. C., Gustafsson, L. E., & Persson, M. G. (1999). Nitric oxide generation, tachyphylaxis and cross-tachyphylaxis from nitrovasodilators in vivo. *European Journal of Pharmacology*, *385*, 137–145. https://doi.org/10.1016/S0014-2999(99)00720-7.

Anand-Srivastava, M. B., Sairam, M. R., & Cantin, M. (1990). Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. Possible coupling of clearance atrial natriuretic factor receptors to adenylate cyclase/cAMP signal transduction system. *Journal of Biological Chemistry*, *265*, 8566–8572. https://doi.org/10.1016/S0021-9258(19)38925-2.

Becker, J. R., Chatterjee, S., Robinson, T. Y., Bennett, J. S., Panakova, D., Galindo, C. L., Zhong, L., Shin, J. T., Coy, S. M., Kelly, A. E., Roden, D. M., Lim, C. C., & MacRae, C. A. (2014). Differential activation of natriuretic peptide receptors modulates cardiomyocyte proliferation during development. *Development*, *141*, 335–345. https://doi.org/10.1242/dev.100370.

Campbell, D. J. (2017). Long-term neprilysin inhibition – Implications for ARNIs. *Nature Reviews Cardiology*, *14*, 171–186. https://doi.org/10.1038/nrcardio.2016.200.

Cataliotti, A., Giordano, M., De Pascale, E., Giordano, G., Castellino, P., Jougasaki, M., Costello, L. C., Boerrigter, G., Tsuruda, T., Belluardo, P., Lee, S. C., Huntley, B., Sandberg, S., Malatino, L. S., & Burnett, J. C. Jr. (2002). CNP production in the kidney

and effects of protein intake restriction in nephrotic syndrome. *American Journal of Physiology. Renal Physiology*, *283*, F464–472. https://doi.org/10.1152/ajprenal.00372.2001.

Charles, C. J., Espiner, E. A., Nicholls, M. G., Richards, A. M., Yandle, T. G., Protter, A., & Kosoglou, T. (1996). Clearance receptors and endopeptidase 24.11: Equal role in natriuretic peptide metabolism in conscious sheep. *American Journal of Physiology*, *271*, R373–380.

D'Agati, V. D., Kaskel, F. J., & Falk, R. J. (2011). Focal segmental glomerulosclerosis. *New England Journal of Medicine*, *365*, 2398–2411. https://doi.org/10.1056/NEJMra1106556.

Davis, J., Burr, A. R., Davis, G. F., Birnbaumer, L., & Molkentin, J. D. (2012). A TRPC6-dependent pathway for myofibroblast transdifferentiation and wound healing in vivo. *Developmental Cell*, *23*, 705–715.

Debiec, H., Guigonis, V., Mougenot, B., Decobert, F., Haymann, J. P., Bensman, A., Deschenes, G., & Ronco, P. M. (2002). Antenatal membranous glomerulonephritis due to anti-neutral endopeptidase antibodies. *New England Journal of Medicine*, *346*, 2053–2060. https://doi.org/10.1056/NEJMoa012895.

Dousa, T. P. (1999). Cyclic-3',5'-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney International*, *55*, 29–62. https://doi.org/10.1046/j.1523-1755.1999.00233.x.

Durand-Arczynska, W., Marmy, N., & Durand, J. (1993). Caldesmon, calponin and alpha-smooth muscle actin expression in subcultured smooth muscle cells from human airways. *Histochemistry*, *100*, 465–471.

El Andalousi, J., Li, Y., & Anand-Srivastava, M. B. (2013). Natriuretic peptide receptor-C agonist attenuates the expression of cell cycle proteins and proliferation of vascular smooth muscle cells from spontaneously hypertensive rats: Role of Gi proteins and MAPkinase/PI3kinase signaling. *PLoS One*, *8*, e76183.

Flannery, P. J., & Spurney, R. F. (2006). Transactivation of the epidermal growth factor receptor by angiotensin II in glomerular podocytes. *Nephron Experimental Nephrology*, *103*, e109–118. https://doi.org/10.1159/000092196.

Francis, S. H., Busch, J. L., Corbin, J. D., & Sibley, D. (2010). cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacological Reviews*, *62*, 525–563. https://doi.org/10.1124/pr.110.002907.

Freedman, N. J., & Lefkowitz, R. J. Desensitization of G protein-coupled receptors. *Recent Progess in Hormone Research*, *51*, 319–351; discussion 352-313, 1996.

Hashimoto, Y., Nakao, K., Hama, N., Imura, H., Mori, S., Yamaguchi, M., Yasuhara, M., & Hori, R. (1994). Clearance mechanisms of atrial and brain natriuretic peptides in rats. *Pharmaceutical Research*, *11*, 60–64.

Hubers, S. A., & Brown, N. J. (2016). Combined angiotensin receptor antagonism and neprilysin inhibition. *Circulation*, *133*, 1115–1124. https://doi.org/10.1161/circulationaha.115.018622

Kitiyakara, C., Eggers, P., & Kopp, J. B. (2004). Twenty-one-year trend in ESRD due to focal segmental glomerulosclerosis in the United States. *American Journal of Kidney Diseases*, *44*, 815–825. https://doi.org/10.1016/S0272-6386(04)01081-9.

Korbet, S. M. (2012). Treatment of primary FSGS in adults. *Journal of the American Society of Nephrology*, *23*, 1769–1776. https://doi.org/10.1681/ASN.2012040389.

Koyama, S., Inoue, T., Terai, T., Takimoto, K., Kato, M., Ito, K., Neya, M., Seki, J., Kobayashi, Y., Kyogoku, Y., & Yoshida, K. (1994). AP-811, a novel ANP-C receptor selective agonist. *International Journal of Peptide and Protein Research*, *43*, 332–336. https://doi.org/10.1111/j.1399-3011.1994.tb00527.x.

Kukkonen, P., Vuolteenaho, O., & Ruskoaho, H. (1992). Basal and volume expansion-stimulated plasma atrial natriuretic peptide concentrations and hemodynamics in conscious rats: Effects of SCH 39.370, an endopeptidase inhibitor, and C-ANF-(4–23), a clearance receptor ligand. *Endocrinology*, *130*, 755–765.

Lee, D. I., Zhu, G., Sasaki, T., Cho, G. S., Hamdani, N., Holewinski, R., Jo, S. H., Danner, T., Zhang, M., Rainer, P. P., Bedja, D., Kirk, J. A., Ranek, M. J., Dostmann, W. R., Kwon, C., Margulies, K. B., Van Eyk, J. E., Paulus, W. J., Takimoto, E., & Kass, D. A. (2015). Phosphodiesterase 9A controls nitric-oxide-independent cGMP and hypertrophic heart disease. *Nature*, *519*, 472–476. https://doi.org/10.1038/nature14332

Lessard, J. L. (1988). Two monoclonal antibodies to actin: One muscle selective and one generally reactive. *Cell Motility and the Cytoskeleton*, *10*, 349–362.

Lewko, B., Endlich, N., Kriz, W., Stepinski, J., & Endlich, K. (2004). C-type natriuretic peptide as a podocyte hormone and modulation of its cGMP production by glucose and mechanical stress. *Kidney International*, *66*, 1001–1008. https://doi.org/10.1111/j.1523-1755.2004.00848.x.

Lewko, B., Golos, M., Latawiec, E., Angielski, S., & Stepinski, J. (2006). Regulation of cGMP synthesis in cultured podocytes by vasoactive hormones. *Journal of Physiology and Pharmacology*, *57*, 599–610.

Li, X., Chuang, P. Y., D'Agati, V. D., Dai, Y., Yacoub, R., Fu, J., Xu, J., Taku, O., Premsrirut, P. K., Holzman, L. B., & He, J. C. (2015). Nephrin preserves podocyte viability and glomerular structure and function in adult kidneys. *Journal of the American Society of Nephrology*, *26*, 2361–2377. https://doi.org/10.1681/ASN.2014040405.

Li, Y., Sarkar, O., Brochu, M., & Anand-Srivastava, M. B. (2014). Natriuretic peptide receptor-C attenuates hypertension in spontaneously hypertensive rats: Role of nitroxidative stress and Gi proteins. *Hypertension*, *63*, 846–855.

Maack, T., Suzuki, M., Almeida, F. A., Nussenzveig, D., Scarborough, R. M., McEnroe, G. A., & Lewicki, J. A. (1987). Physiological role of silent receptors of atrial natriuretic factor. *Science*, *238*, 675–678. https://doi.org/10.1126/science.2823385.

Makino, H., Mukoyama, M., Mori, K., Suganami, T., Kasahara, M., Yahata, K., Nagae, T., Yokoi, H., Sawai, K., Ogawa, Y., Suga, S., Yoshimasa, Y., Sugawara, A., Tanaka, I., & Nakao, K. (2006). Transgenic overexpression of brain natriuretic peptide prevents the progression of diabetic nephropathy in mice. *Diabetologia*, *49*, 2514–2524. https://doi.org/10.1007/s00125-006-0352-y.

Maurice, D. H., Ke, H., Ahmad, F., Wang, Y., Chung, J., & Manganiello, V. C. (2014). Advances in targeting cyclic nucleotide phosphodiesterases. *Nature Reviews Drug Discovery*, *13*, 290–314. https://doi.org/10.1038/nrd4228.

Murthy, K. S., Teng, B., Jin, J., & Makhlouf, G. M. (1998). G protein-dependent activation of smooth muscle eNOS via natriuretic peptide clearance receptor. *American Journal of Physiology*, *275*, C1409–1416. https://doi.org/10.1152/ajpcell.1998.275.6.C1409.

Nishizawa, N., Nakamura, G., Noguchi, Y., Nakagawa, H., Shimizu, A., Nakayama, M., Takekawa, S., & Asami, T. (2017). A potent and selective natriuretic peptide receptor-3 blocker 11-mer peptide created by hybridization of musclin and atrial natriuretic peptide. *Bioorganic & Medicinal Chemistry Letters*, *27*, 3542–3545. https://doi.org/10.1016/j.bmcl.2017.05.061.

Ogawa, Y., Mukoyama, M., Yokoi, H., Kasahara, M., Mori, K., Kato, Y., Kuwabara, T., Imamaki, H., Kawanishi, T., Koga, K., Ishii, A., Tokudome, T., Kishimoto, I., Sugawara, A., & Nakao, K. (2012). Natriuretic peptide receptor guanylyl cyclase-A protects podocytes from aldosterone-induced glomerular injury. *Journal of the American Society of Nephrology*, *23*, 1198–1209. https://doi.org/10.1681/ASN.2011100985.

Okolicany, J., McEnroe, G. A., Koh, G. Y., Lewicki, J. A., & Maack, T. (1992). Clearance receptor and neutral endopeptidase-mediated metabolism of atrial natriuretic factor. *American Journal of Physiology*, *263*, F546–553. https://doi.org/10.1152/ajprenal.1992.263.3.F546.

Pandey, K. N. (2014). Guanylyl cyclase/natriuretic peptide receptor-A signaling antagonizes phosphoinositide hydrolysis, Ca(2+) release, and activation of protein kinase C. *Frontiers in Molecular Neuroscience*, *7*, 75. https://doi.org/10.3389/fnmol.2014.00075.

Park, J., Shrestha, R., Qiu, C., Kondo, A., Huang, S., Werth, M., Li, M., Barasch, J., & Susztak, K. (2018). Single-cell transcriptomics of the mouse kidney reveals potential cellular targets of kidney disease. *Science*, *360*, 758–763. https://doi.org/10.1126/science.aar2131.

Potter, L. R. (2011). Natriuretic peptide metabolism, clearance and degradation. *FEBS Journal*, *278*, 1808–1817. https://doi.org/10.1111/j.1742-4658.2011.08082.x.

Potter, L. R., Abbey-Hosch, S., & Dickey, D. M. (2006). Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocrine Reviews*, *27*, 47–72. https://doi.org/10.1210/er.2005-0014

Ren, S., Xin, C., Beck, K. F., Saleem, M. A., Mathieson, P., Pavenstadt, H., Pfeilschifter, J., & Huwiler, A. (2005). PPARalpha activation upregulates nephrin expression in human embryonic kidney epithelial cells and podocytes by a dual mechanism. *Biochemical and Biophysical Research Communications*, *338*, 1818–1824.

Ristola, M., Arpiainen, S., Shimokawa, T., Ra, C., Tienari, J., Saleem, M. A., Holthofer, H., & Lehtonen, S. (2013). Regulation of nephrin gene by the Ets transcription factor, GA-binding Protein. *Nephrology Dialysis Transplantation*, *28*, 846–855. https://doi.org/10.1093/ndt/gfs482.

Robins, R., Baldwin, C., Aoudjit, L., Cote, J. F., Gupta, I. R., & Takano, T. (2017). Rac1 activation in podocytes induces the spectrum of nephrotic syndrome. *Kidney International*, *92*, 349–364.

Rose, R. A., & Giles, W. R. (2008). Natriuretic peptide C receptor signalling in the heart and vasculature. *Journal of Physiology*, *586*, 353–366. https://doi.org/10.1113/jphysiol.2007.144253.

Rosenberg, A. Z., & Kopp, J. B. (2017). Focal segmental glomerulosclerosis. *Clinical Journal of the American Society of Nephrology*, *12*, 502–517. https://doi.org/10.2215/CJN.05960616.

Rotella, D. P. (2002). Phosphodiesterase 5 inhibitors: Current status and potential applications. *Nature Reviews Drug Discovery*, *1*, 674–682.

Sagare, A. P., Bell, R. D., Zhao, Z., Ma, Q., Winkler, E. A., Ramanathan, A., & Zlokovic, B. V. (2013). Pericyte loss influences Alzheimer-like neurodegeneration in mice. *Nature Communications*, *4*, 2932. https://doi.org/10.1038/ncomms3932.

Schwartz, E. J., Cara, A., Snoeck, H., Ross, M. D., Sunamoto, M., Reiser, J., Mundel, P., & Klotman, P. E. (2001). Human immunodeficiency virus-1 induces loss of contact inhibition in podocytes. *Journal of the American Society of Nephrology*, *12*, 1677–1684. https://doi.org/10.1681/ASN.V1281677.

Shen, K., Johnson, D. W., & Gobe, G. C. (2016). The role of cGMP and its signaling pathways in kidney disease. *American Journal of Physiology–renal Physiology*, *311*, F671–F681. https://doi.org/10.1152/ajprenal.00042.2016.

Staffel, J., Valletta, D., Federlein, A., Ehm, K., Volkmann, R., Fuchsl, A. M., Witzgall, R., Kuhn, M., & Schweda, F. (2017). Natriuretic peptide receptor guanylyl cyclase-A in podocytes is renoprotective but dispensable for physiologic renal function. *Journal of the American Society of Nephrology*, *28*, 260–277. https://doi.org/10.1681/ASN.2015070731.

Suganami, T., Mukoyama, M., Sugawara, A., Mori, K., Nagae, T., Kasahara, M., Yahata, K., Makino, H., Fujinaga, Y., Ogawa, Y., Tanaka, I., & Nakao, K. (2001). Overexpression of brain natriuretic peptide in mice ameliorates immune-mediated renal injury. *Journal of the American Society of Nephrology*, *12*, 2652–2663. https://doi.org/10.1681/ASN.V12122652.

Tapia, E., Sanchez-Lozada, L. G., Soto, V., Manrique, A. M., Ortiz-Vega, K. M., Santamaria, J., Medina-Campos, O. N., Cristobal, M., Avila-Casado, C., Pedraza-Chaverri, J., Rodriguez-Iturbe, B., & Franco, M. (2012). Sildenafil treatment prevents glomerular hypertension and hyperfiltration in rats with renal ablation. *Kidney and Blood Pressure Research*, *35*, 273–280. https://doi.org/10.1159/000334952.

Theilig, F., Bostanjoglo, M., Pavenstadt, H., Grupp, C., Holland, G., Slosarek, I., Gressner, A. M., Russwurm, M., Koesling, D., & Bachmann, S. (2001). Cellular distribution and function of soluble guanylyl cyclase in rat kidney and liver. *Journal of the American Society of Nephrology*, *12*, 2209–2220. https://doi.org/10.1681/ASN.V12112209.

Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., & Brown, R. A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nature Reviews Molecular Cell Biology*, *3*, 349–363. https://doi.org/10.1038/nrm809.

Veale, C. A., Alford, V. C., Aharony, D., Banville, D. L., Bialecki, R. A., Brown, F. J., Damewood, J. R. Jr., Dantzman, C. L., Edwards, P. D., Jacobs, R. T., Mauger, R. C., Murphy, M. M., Palmer, W., Pine, K. K., Rumsey, W. L., Garcia-Davenport, L. E., Shaw, A., Steelman, G. B., Surian, J. M., & Vacek, E. P. (2000). The discovery of non-basic atrial natriuretic peptide clearance receptor antagonists. Part 1. *Bioorganic & Medicinal Chemistry Letters*, *10*, 1949–1952. https://doi.org/10.1016/S0960-894X(00)00387-5.

Wang, L., Chang, J. H., Buckley, A. F., & Spurney, R. F. (2019). Knockout of TRPC6 promotes insulin resistance and exacerbates glomerular injury in Akita mice. *Kidney International*, *95*, 321–332. https://doi.org/10.1016/j.kint.2018.09.026.

Wang, L., Ellis, M. J., Gomez, J. A., Eisner, W., Fennell, W., Howell, D. N., Ruiz, P., Fields, T. A., & Spurney, R. F. (2012). Mechanisms of the proteinuria induced by Rho GTPases. *Kidney International*, *81*, 1075–1085. https://doi.org/10.1038/ki.2011.472.

Wang, L., Jirka, G., Rosenberg, P. B., Buckley, A. F., Gomez, J. A., Fields, T. A., Winn, M. P., & Spurney, R. F. (2015). Gq signaling causes glomerular injury by activating TRPC6. *Journal of Clinical Investigation*, *125*, 1913–1926. https://doi.org/10.1172/JCI76767.

Wang, L., Sha, Y., Bai, J., Eisner, W., Sparks, M. A., Buckley, A. F., & Spurney, R. F. (2017). Podocyte-specific knockout of cyclooxygenase 2 exacerbates diabetic kidney disease. *American Journal of Physiology–renal Physiology*, *313*, F430–F439. https://doi.org/10.1152/ajprenal.00614.2016.

Wegner, M., Stasch, J. P., Hirth-Dietrich, C., Dressel, J., Voges, K. P., & Kazda, S. (1995). Interaction of a neutral endopeptidase inhibitor with an ANP-C receptor ligand in anesthetized dogs. *Clinical and Experimental Hypertension*, *17*, 861–876. https://doi.org/10.3109/10641969509033640.

Whitesall, S. E., Hoff, J. B., Vollmer, A. P., & D'Alecy, L. G. (2004). Comparison of simultaneous measurement of mouse systolic arterial blood pressure by radiotelemetry and tail-cuff methods. *American Journal of Physiology. Heart and Circulatory Physiology*, *286*, H2408–2415. https://doi.org/10.1152/ajpheart.01089.2003.

Wiggins, R. C. (2007). The spectrum of podocytopathies: A unifying view of glomerular diseases. *Kidney International*, *71*, 1205–1214.

William, M., Hamilton, E. J., Garcia, A., Bundgaard, H., Chia, K. K., Figtree, G. A., & Rasmussen, H. H. (2008). Natriuretic peptides stimulate the cardiac sodium pump via NPR-C-coupled NOS activation. *American Journal of Physiology. Cell Physiology*, *294*, C1067–1073. https://doi.org/10.1152/ajpcell.00243.2007.

Wilson, P. C., Wu, H., Kirita, Y., Uchimura, K., Ledru, N., Rennke, H. G., Welling, P. A., Waikar, S. S., & Humphreys, B. D. (2019). The single-cell transcriptomic landscape of early human diabetic nephropathy. *Proceedings of the National Academy of Sciences of the USA*, *116*, 19619–19625. https://doi.org/10.1073/pnas.1908706116.

Wolf, G., Chen, S., & Ziyadeh, F. N. (2005). From the periphery of the glomerular capillary wall toward the center of disease: Podocyte injury comes of age in diabetic nephropathy. *Diabetes*, *54*, 1626–1634.

Wong, D. W. L., Yiu, W. H., Chan, K. W., Li, Y., Li, B., Lok, S. W. Y., Taketo, M. M., Igarashi, P., Chan, L. Y. Y., Leung, J. C. K., Lai, K. N., & Tang, S. C. W. (2018). Activated renal tubular Wnt/beta-catenin signaling triggers renal inflammation during overload proteinuria. *Kidney International*, *93*, 1367–1383.

Yamauchi, K., Takano, Y., Kasai, A., Hayakawa, K., Hiramatsu, N., Enomoto, N., Yao, J., & Kitamura, M. (2006). Screening and identification of substances that regulate nephrin gene expression using engineered reporter podocytes. *Kidney International*, *70*, 892–900. https://doi.org/10.1038/sj.ki.5001625.

Zhu, L., Jiang, R., Aoudjit, L., Jones, N., & Takano, T. (2011). Activation of RhoA in podocytes induces focal segmental glomerulosclerosis. *Journal of the American Society of Nephrology*, *22*, 1621–1630. https://doi.org/10.1681/ASN.2010111146.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Wang, L., Tang, Y., Buckley, A. F., & Spurney, R. F. (2021). Blockade of the natriuretic peptide clearance receptor attenuates proteinuria in a mouse model of focal segmental glomerulosclerosis. *Physiological Reports*, 9, e15095. https://doi.org/10.14814/phy2.15095